

Article

Assessing Microplastic Contamination in Zooplanktonic Organisms from Two River Estuaries

Francisca Espincho ^{1,2,*}, Rúben Pereira ^{1,2}, Sabrina M. Rodrigues ^{1,2} , Diogo M. Silva ^{1,2}, C. Marisa R. Almeida ^{2,3} and Sandra Ramos ^{2,4}

¹ ICBAS—Institute of Biomedical Sciences Abel Salazar, Porto University, Rua de Jorge Viterbo Ferreira n° 228, 4050-313 Porto, Portugal; smagalhaes@ciimar.up.pt (S.M.R.)

² CIIMAR—Interdisciplinary Centre of Marine and Environmental Research, Porto University, Terminal de Cruzeiros do Porto de Leixões, Avenida General Norton de Matos, S/N, 4450-208 Matosinhos, Portugal; calmeida@fc.up.pt (C.M.R.A.)

³ Chemistry and Biochemistry Department, Sciences Faculty, Porto University, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal

⁴ Biology Department, Sciences Faculty, Porto University, Rua do Campo Alegre, 687, 4169-007 Porto, Portugal

* Correspondence: mespincho@ciimar.up.pt

Abstract: The present work aims to evaluate the MP contamination of zooplankton and its impact on MP trophic transfers at the lower levels of the food web in a field study. During 1 year, seasonal surveys were conducted to collect zooplankton and water samples from different sites in two estuaries, the Douro and Lima estuaries (NW, Portugal). The zooplankton was quantified and identified into major zooplanktonic groups. Dedicated protocols that had been previously optimized were used to assess the MP presence in the water samples and in two of the most abundant zooplankton groups (copepods and chaetognaths). The results showed the presence of MPs in all water samples, with similar MP concentrations in both estuaries (Lima: 2.4 ± 2.0 MPs m^{-3} ; Douro: 2.3 ± 1.9 MPs m^{-3}). In general, no temporal or spatial variations were observed. Fibres, blue and of a small size (<1 mm), were the most common characteristics of the MPs found in the water and zooplankton, indicating that water can be a source of MPs for zooplankton. Chaetognatha exhibited higher MP contamination in the Lima (2.9 ± 3.1 MPs ind^{-1}) and Douro (2.0 ± 2.8 MPs ind^{-1}) estuaries than Copepoda, which tended to have lower levels of MP contamination (Lima: 0.95 ± 1.12 MPs ind^{-1} ; Douro: 1.1 ± 1.2 MPs ind^{-1}). Such differences in the MP concentrations between these two categories of zooplanktonic organisms indicate a possible MP trophic transfer at the lower levels of the food web. The results highlight the novel possibility of an MP trophic transfer in zooplankton and the need to fully assess the impacts of MPs in real scenarios.

Keywords: microplastics; zooplankton; estuaries; Copepoda; Chaetognatha



Citation: Espincho, F.; Pereira, R.; Rodrigues, S.M.; Silva, D.M.; Almeida, C.M.R.; Ramos, S. Assessing Microplastic Contamination in Zooplanktonic Organisms from Two River Estuaries. *Water* **2024**, *16*, 992. <https://doi.org/10.3390/w16070992>

Academic Editor: Marina Marcella Manca

Received: 1 March 2024

Revised: 22 March 2024

Accepted: 26 March 2024

Published: 29 March 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Plastic is a major concern in marine environments, and is the most abundant type of marine debris [1]. Microplastics (MPs) have been receiving increased attention from the scientific community in recent years, since their presence is extremely significant in marine pollution, accounting for 92% of the plastic debris found on the ocean surface [2]. MPs are commonly defined as plastic debris with a diameter of less than 5 mm [3], and are disseminated all over the globe. MPs have been detected in distinct ecosystems, both in marine ecosystems, such as beaches [4] and seabed sediments [5], and in freshwater ecosystems [6], and even in the atmosphere [7]. MPs are transported to different regions and ecosystems, even where the presence of humans is significantly reduced. Transitional environments between marine and freshwater environments, such as estuaries, are also affected by MPs [8–10]. The concentration of MPs in these environments is fairly variable and depends on several factors, such as the wind and current conditions, the geographical

characteristics, the presence of urban areas and shipping trade routes [11], and the proximity to wastewater treatment plants (WWTPs) [12] and to abandoned, lost, or discarded fishing gear (ALDFGs) [13]. MPs are more frequently found in enclosed or semi-enclosed sea areas, and in the upper levels of the water column, near the surface water and shorelines. MPs can also sink and concentrate at the bottom of the aquatic environment due to modifications in their density and buoyancy [11].

Estuaries are also contaminated with MPs. Estuaries are important aquatic ecosystems, since they represent a transitional zone between the sea and freshwater streams, playing an important role for several species and providing important ecosystem services [10,14]. Estuaries also provide an important tool for understanding the dispersion mechanisms of MPs [8]. In recent years, we have seen an increase in research focused on the presence and abundance of MPs in estuaries [9,10,14–18].

Estuarine communities are composed of a few resident species and several migratory species that temporarily inhabit these areas, and the presence of MPs can affect all of them. Zooplankton represent the animal component of planktonic communities, and they are often defined as organisms that are carried by tides and currents, and are not able to swim or move willingly against them [19]. Zooplankton are present in a great variety of aquatic environments, and they occupy a key place in the food web by connecting two trophic levels, small primary producers and large consumers [20–22]. A perturbation of the zooplankton biomass can disturb and impact the biomass stocks of other types of plankton, and influence ecosystem services [23]. As highlighted by Rodrigues et al. [24], the association between MPs and plankton is still relatively underrepresented when considering the number of publications on MPs. Furthermore, several of these publications are laboratory studies, and comparisons between them and field studies must be made with caution, due to the differences in the MP concentration, polymer type, shape, and MP condition (laboratory studies tend to use virgin MPs—MPs that have not been exposed to environmental conditions) [24]. The ingestion of MPs by zooplankton has been verified in a few recent field studies, showing that zooplankton tend to ingest MPs that are present in their environment and are of a similar size to their typical prey [9,18,25,26]. Laboratory studies have reported many biological impacts upon ingestion, such as internal injuries to body tissues and the alimentary tract [23,27], impaired feeding behaviour [28], reduced fecundity, and reduced energy levels, leading to deficient growth [28,29]. In addition, waterborne pollutants are able to adhere to the plastic polymer of many MPs and may induce chemical toxicity in zooplankton [16,30]. It has also been shown in laboratory studies that the ageing and weathering action that MPs withstand during their long-term permanence in aquatic environments can lead to an increase in their uptake by certain organisms. Vroom et al. [31] confirmed that aged MPs were ingested by more individuals and at faster rates than pristine MPs in planktonic Copepoda, and this higher ingestion rate could be due to differences in shapes and biofouling that lead to a similarity between these MPs and the typical food items of these organisms. In terms of ecological effects, the number of studies is even lower. For example, Setälä et al. [32] showed in a laboratory study that MPs ingested by zooplankton have the potential to be transferred to higher trophic levels along the food webs, through the ingestion of zooplankton by a predator. In addition, Sipps et al. [9] stressed that MPs may be incorporated into faecal pellets, sinking into the water column and changing the bioavailability of otherwise-buoyant MPs. But more research is needed, namely regarding field studies, to take into consideration the real conditions to which the organisms are exposed.

Considering the emergent concern of MP pollution, it is important to increase the scientific knowledge of the effect of MPs on aquatic environments and organisms, and further our understanding on the real impacts of MPs supported by field data, particularly from lower trophic levels such as zooplankton. Therefore, the main goal of this study was to assess the MP contamination of estuarine zooplankton using two Portuguese estuaries as case studies, namely the Douro and Lima estuaries (NW Portugal), to specifically achieve the following: (1) assess the temporal and spatial patterns of MPs and zooplankton ratios in

the two distinct estuaries, and (2) investigate the occurrence of MPs in two relevant groups of zooplankton organisms, namely copepods and chaetognaths, which can have an impact on MP trophic transfers at the lower levels of the food web.

2. Materials and Methods

2.1. Study Area

Two distinct estuaries in the north of Portugal were selected as case studies: the Douro River estuary and the Lima River estuary (Figure 1). The Douro estuary is a salt-wedge estuary, and its upstream limit is defined by the Crestuma dam, located 21.6 km upstream of the river mouth [33]. The Douro estuary can be divided into three distinct zones [34]: the lower, middle, and upper estuary. The Douro estuary is characterized by a heavy urban presence, mostly along the last 8 km of its length, which harbours two major Portuguese cities [35]. It is also heavily influenced by the wastewater treatment plants' effluents and rivers/streams that drain into the estuary [17,35]. The Lima estuary is a seasonally stratified estuary, and it is also divided into three areas: the narrow lower estuary, located in the river mouth; the middle estuary, which is classified as a shallow saltmarsh zone; and the upper estuary, characterized by a decrease in depth and channel width [36]. The Lima estuary is less impacted by anthropogenic activities, but still with some urban pressure and a large commercial harbour in the lower estuary [37]. This estuary still has natural banks and a large saltmarsh area located in the middle estuary, and upstream, the Lima estuary receives urban and agricultural effluents [38].

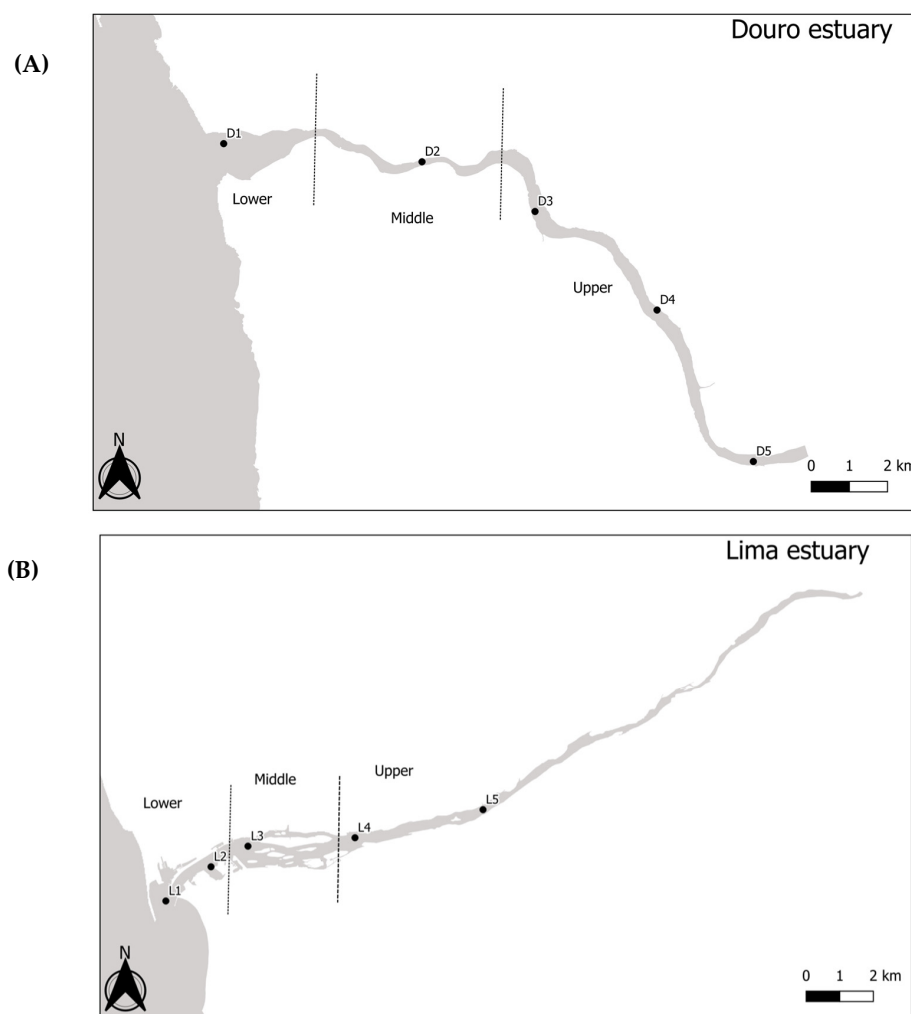


Figure 1. Locations of the five sampling stations in the Douro estuary (A) and Lima estuary (B), distributed throughout the lower, middle, and upper sections of the estuaries.

2.2. Sampling Methodology

Sampling campaigns were conducted in 2022 in the following months: February (winter campaign), May (spring campaign), August (summer campaign), and November (autumn campaign). Due to logistic constraints with the vessel used in the sampling surveys, it was not possible to conduct the summer campaign in the Lima estuary. In each estuary, five sampling stations distributed across the horizontal gradient were surveyed, covering the lower, middle, and upper sections of each estuary (Figure 1). In the Douro estuary, D1 was located in the lower estuary near the river mouth; D2 was located in the middle estuary; and D3, D4, and D5 were located in the upper estuary. In the Lima estuary, L1 and L2 were located in the lower estuary; L3 was located in the middle estuary; and L4 and L5 were located in the upper estuary. Zooplankton and MPs in estuarine water were collected by means of a 150 μm mesh size planktonic net (in general, the standard size regarding zooplankton sampling is a mesh size between 150 and 200 μm [39,40]). At each sampling site, planktonic tows were performed for 1 min near the surface of the water. The samples were immediately preserved with 70% ethanol until further laboratory analyses. The volume of filtered water was quantified with a flowmeter (Hydro-Bios) attached to the plankton net.

2.3. Zooplankton Analysis—Quantification and Identification of Major Zooplanktonic Groups

The quantification and identification of major zooplanktonic groups in the samples were performed by sub-sampling 2 mL of the original sample in a Bogorov chamber and analysing it using a stereomicroscope. This procedure was performed three times in total, each time sub-sampling 2 mL of each sample. The number of zooplankton organisms was standardized to the number of individuals per m^3 of filtered water. The major zooplanktonic groups considered were as follows: Copepoda, Copepoda and Cirripedia nauplii, Cladocera, *Oikopleura*, Cirripedia cypris larvae, Tintinnina, Hydrozoa, Chaetognatha, fish eggs, Ostracoda, veligers, fish larvae, and polychaete larvae [41]. All preventive measures to prevent MP contamination, as detailed in the following section, were carried out in all zooplankton laboratory analyses.

2.4. MP Analysis

2.4.1. Measures to Prevent MP Contamination

The prevention of MP contamination was of key importance throughout the course of this study. Several measures were taken to ensure no MP contamination occurred in any samples: specific lab coats of cotton were always used during laboratory procedures; all laboratory materials and supplies were thoroughly washed with deionized water and ethanol before use; and for procedures on the stereomicroscope with open samples, an open Petri dish with deionized water was placed near the stereomicroscope and inspected for MPs at the beginning and end of the procedures.

2.4.2. MPs in Water Samples

An MP analysis was executed through a protocol previously developed by the team, adapted from the NOAA protocol, and described by Rodrigues et al. [42]. In summary, the samples were initially sieved through a 0.03 mm filter cloth and the solids were placed on a beaker, with both previously washed with deionized water. The samples were left to dry overnight at 90 °C. The following day, 20 mL of a 0.05 M Fe(II) solution and 20 mL of a 30% H_2O_2 solution were added to each sample, and the samples were then heated at 75 °C. Twenty minutes after the occurrence of a chemical reaction (in the form of heat and the appearance of bubbles), another 20 mL of a 30% H_2O_2 solution was added. After another waiting period of 20 min for a second chemical reaction to occur, 18 g of NaCl was added in order to increase the density of the solution. The samples continued to be heated for another 30 min. Later, the saturated solution was placed on a density separator and left overnight. The next day, the solids floating on the density separator were filtered and left to dry at room temperature.

2.4.3. MPs in Zooplanktonic Organisms

MPs were retrieved from the zooplankton using an adaptation of a dedicated protocol developed to analyse MPs in planktonic organisms, including zooplankton [43]. The basis of the process consisted of digesting the organic content of the organism with a 30% H₂O₂ solution. The protocol was properly optimized and validated for zooplankton samples through several tests, such as laboratory tests to assess possible sources of contamination, tests with several types of common MP polymers (polyethylene (PE), polyvinyl chloride (PVC), polyethylene terephthalate /polyester (PET), cellulose acetate, rayon, and polymethyl methacrylate (PMMA)), and tests to determine the ideal exposure time. It was concluded that the ideal exposure time to a 30% H₂O₂ solution was 7 h [43].

Two groups of zooplankton were selected to assess the MP contamination in zooplanktonic organisms, namely Copepoda (mostly composed of Calanoida) and Chaetognatha. These two groups were chosen because they are typically frequent and abundant in temperate estuarine zooplankton populations. Also, they represent different trophic levels, as copepods are mostly composed of primary consumers and chaetognaths are mainly predators [44,45]. First, each individual was carefully separated and inspected for any MPs or inorganic components on its exterior. After a thorough cleanse of each individual with deionized water, the organisms were placed in a clean glass flask. Three replicates were prepared for each sampling station from each sampling campaign. The number of zooplankton organisms selected per sample was 30 individuals. However, in some samples, a lower number was available due to different reasons; for example, the organisms were absent during that specific time of the year, or the organisms were not in a well-preserved condition, probably due to sample preservation issues (February: L1 = 1 chaetognath, L2 = 24 copepods, L3 = 4 copepods, L4 = 21 copepods, L5 = 14 copepods, C3 = 3 copepods, and C4 = 14 copepods; May: L2 = 20 copepods, L3 = 3 chaetognaths, L4 = 9 copepods, L5 = 19 copepods, D1 = 10 chaetognaths, and D2 = 4 copepods; August: D1 = 26 copepods, D1 = 3 chaetognaths, D2 = 2 copepods, D3 = 1 copepod, D4 = 1 copepod, D4 = 1 chaetognath, D5 = 1 copepod, and D5 = 1 chaetognath; November: L1 = 7 chaetognaths, L2 = 19 copepods, L2 = 17 chaetognaths, L3 = 23 copepods, L3 = 10 chaetognaths, L4 = 6 chaetognaths, L5 = 1 copepod, L5 = 2 chaetognaths, D2 = 7 copepods, D2 = 27 chaetognaths, D3 = 3 copepods, D3 = 5 chaetognaths, D5 = 1 copepod, and D5 = 1 chaetognath). Then, 2 mL of a 30% H₂O₂ solution was added to each flask, and the flasks were placed at 65 °C for seven hours to ensure the total digestion of the organic content. Afterwards, the samples were filtered, and the filters were left to dry at room temperature.

2.4.4. MP Characterization and Polymer Analysis

The MPs recovered from each sample of water and zooplanktonic organisms were observed under the stereomicroscope, to be characterized and quantified by their size, shape (fibre, fragment, and film), and colour. A subsample of the most representative particles observed was selected for a further FTIR (Fourier-transform infrared spectroscopy) analysis for polymer identification (around 10% of the total), in accordance with the recommendation of the European Union's Marine Strategy Framework Directive (MSFD) that a proportion (10%) of all samples should be routinely checked to confirm the accuracy of visual examination [46]. The polymer spectra were registered in a PerkinElmer (Waltham, MA, USA) FT-IR Spectrum 2 instrument coupled with attenuated total reflectance (ATR), with a detection limit as small as 10 µm. The FTIR spectra were recorded using an average of 4 scans in the range of 4000–800 cm^{−1}. When necessary, the number of scans recorded in the FTIR spectrum was increased to 20 scans per MP. The obtained spectra were compared with equipment library spectra, and according to Rodrigues et al. [47], matches with confidence levels ≥75% were accepted, after the visual confirmation of each spectrum.

2.5. Data Analysis

For the water samples, the MP abundance was standardized by calculating the number of particles per m^{−3} of water filtered. And, for zooplankton, the MP abundance was

estimated by calculating the number of particles per individual organism. To investigate the ecological impacts of MP contamination in zooplankton, a ratio between the MP concentration in the water samples and the zooplankton abundance was calculated. The zooplankton abundance was transformed by $\log_{10}$ ("zooplankton abundance" + 1) due to the large scale difference between the two data sets, following a similar method to that used by Sun et al. [22].

A one-way analysis of variance (ANOVA) was used to investigate significant differences in the zooplankton abundance, MP concentration in estuarine waters, and MP concentration in Copepoda and Chaetognatha. These differences were investigated by considering the estuaries, sampling months (temporal variations), and sampling stations (spatial variations) as fixed factors. The ANOVA assumptions were tested; namely, the homogeneity of variance was tested with the Cochran test. Whenever necessary, the data were log-transformed to follow the ANOVA assumptions. And, in the case when it was not possible to fulfil the ANOVA assumptions, the non-parametric Kruskal–Wallis test was used. The post-hoc analyses were performed with Fisher's LSD. A significance level of 0.05 was considered for all analyses. All tests were performed with the TIBCO Statistica™ 14.0 software.

3. Results

3.1. Estuarine Zooplankton

The total abundance of zooplankton was, on average, $7.7 \times 10^4 \pm 8.8 \times 10^4$ No m^{-3} in the Douro estuary and $9.2 \times 10^4 \pm 1.0 \times 10^4$ No m^{-3} in the Lima estuary (Figure 2A), without significant differences between the estuaries (one-way ANOVA: $F = 0.12$; $p \geq 0.05$). Also, the composition of the zooplankton community was similar between the two estuaries, with a total of thirteen zooplankton groups identified in both estuaries. In the Douro estuary, 10 groups were identified: Copepoda (mostly composed of Calanoida), Copepoda and Cirripedia naupili, *Oikopleura*, Cirripedia cypris larvae, Tintinnina, Hydrozoa, Chaetognatha, fish eggs, veligers, and fish larvae (Figure 2B); in the Lima estuary, 9 groups were identified: Copepoda, Copepoda and Cirripedia naupili, Cirripedia cypris larvae, Hydrozoa, Chaetognatha, fish eggs, Ostracoda, veligers, and Polychaeta larvae (Figure 2C). Overall, Copepoda was the most common group in all the samples from the two estuaries. However, in May 2022, Cladocera was the most abundant group observed in the Douro estuary (Figure 2B). Copepoda made up between 74% and 90% of the total zooplankton in the three sampling campaigns in the Lima estuary, and between 43% and 84% of the total zooplankton in the four sampling campaigns in the Douro estuary. Nauplii were generally the second most abundant group in both estuaries. Copepoda and nauplii were the only two groups found in all the samples, while the other groups varied among months or between estuaries. *Oikopleura*, Cladocera, and Chaetognatha were also frequently observed, while the remaining groups were less abundant and frequent.

Each estuary presented specific temporal and spatial patterns of zooplankton abundance. In the Douro estuary, there were no significant differences in the temporal (one-way ANOVA: $F = 0.84$; $p \geq 0.05$) or spatial (one-way ANOVA: $F = 1.36$; $p \geq 0.05$) patterns (Figure 2D). Although not significant, there was a tendency for the zooplankton abundance to be lower in the middle estuary and higher in the lower estuary. In November 2022, the zooplankton abundance in the Douro estuary contradicted this general tendency, being higher in the upper estuary, mainly at sites D4 and D5. There was an increase in the zooplankton abundance of site D5 in May, reaching the highest value observed in the Douro estuary.

In contrast, in the Lima estuary, the zooplankton abundance varied significantly among sampling stations (one-way ANOVA: $F = 5.47$; $p < 0.05$), but not among seasons (one-way ANOVA: $F = 0.84$; $p \geq 0.05$) (Figure 2E). A significantly lower zooplankton abundance was observed for the lower estuary in all the sampling campaigns, and higher abundances were observed in the upper section, namely at site L4, which had the highest zooplankton abundance in all the campaigns.

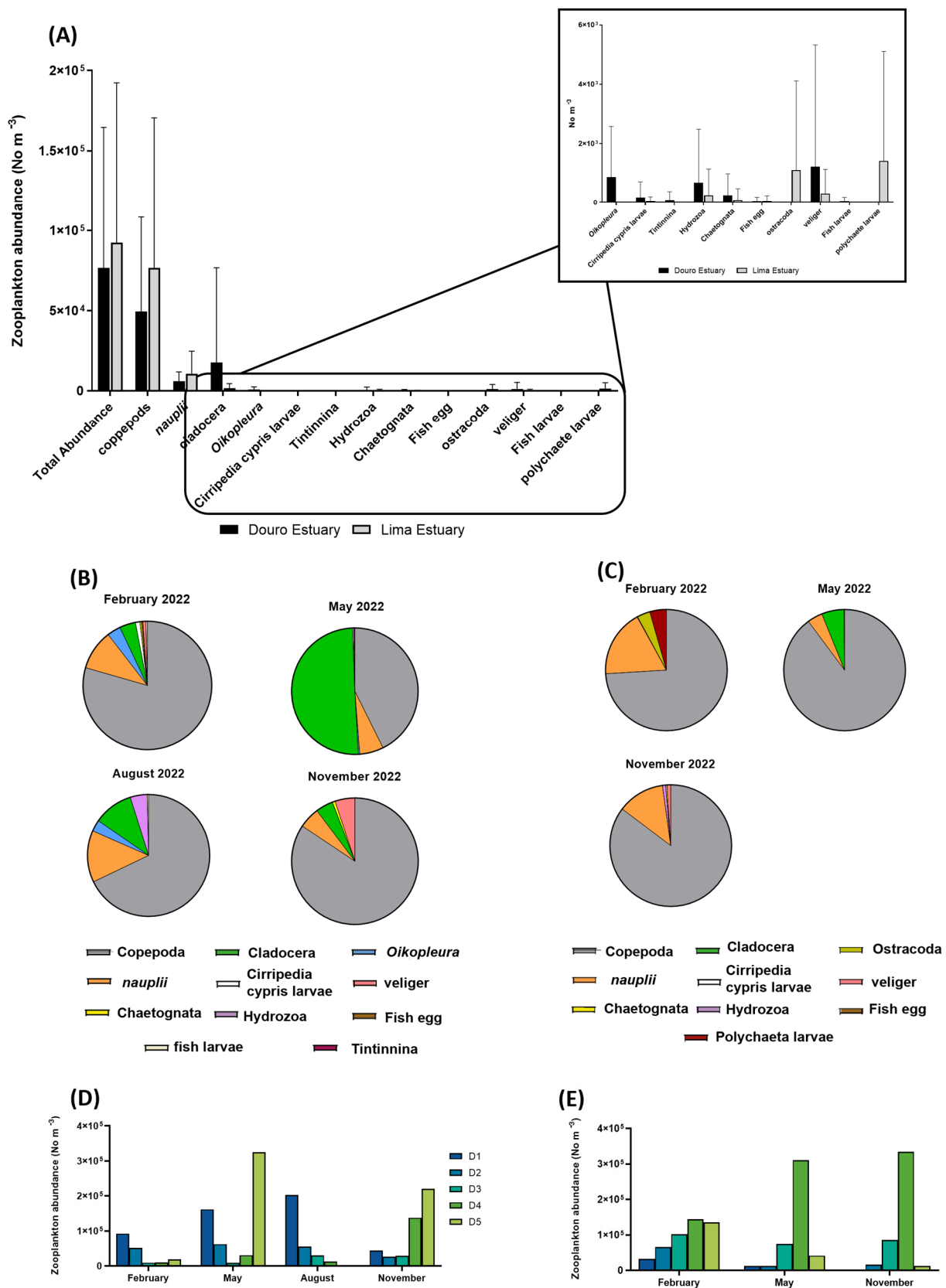


Figure 2. Zooplankton communities of the Douro and Lima estuaries: **(A)** total abundance (No m⁻³) of zooplankton; **(B,C)** monthly composition of the different zooplanktonic groups; and **(D,E)** temporal and spatial variation in zooplankton abundance in the Douro estuary and Lima estuary, respectively.

3.2. MPs in Water Samples

No aerial MP contamination was observed during the sample collection, laboratory procedures, MP characterization, or polymer analysis.

3.2.1. Spatial and Temporal Variation in MPs in Water Samples

A total of 1222 MPs were retrieved from the 20 water samples collected in the Douro estuary and the 15 samples from the Lima estuary. MPs were present in all the samples, with a mean concentration of $2.3 \pm 1.9 \text{ MP m}^{-3}$ in the Douro estuary and $2.4 \pm 2.0 \text{ MP m}^{-3}$ in the Lima estuary. There were no statistical differences in the MP concentration between the two estuaries (one-way ANOVA: $F = 0.065$; $p \geq 0.05$). In the Douro estuary, the maximum MP concentration was observed in November at site D1, reaching 9.6 MP m^{-3} (Figure 3A), although there were no significant temporal (one-way ANOVA: $F = 2.30$; $p \geq 0.05$) or spatial (one-way ANOVA: $F = 0.26$; $p \geq 0.05$) differences in the MP contamination. In the Lima estuary, the MP concentration did not significantly vary spatially (Kruskal–Wallis: $H_4 = 15$; $p \geq 0.05$); however, the MP contamination varied significantly among months, with a significantly higher concentration in November (one-way ANOVA: $F = 6.77$; $p < 0.05$) (Figure 3B).

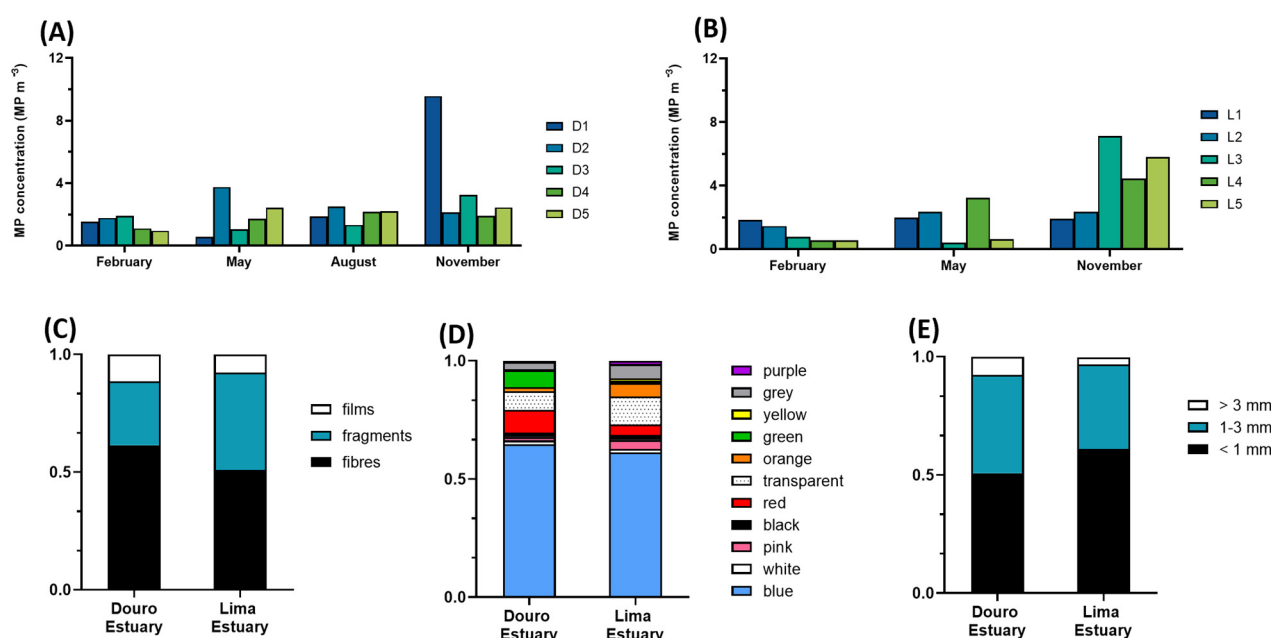


Figure 3. Temporal and spatial variation in MP concentration (MP m^{-3}) in water samples from the Douro estuary (A) and Lima estuary (B). (C–E) represent the distribution of MPs by shape, colour, and size, respectively, in the two estuaries.

3.2.2. Characterization of MPs in Water Samples

The shapes of the MPs found in both estuaries were similar, including fibres, fragments, and films. In both estuaries, fibres were the most common MP shape retrieved from the water samples, accounting for 61% of the total MPs found in the Douro estuary and 51% of the total MPs collected in the Lima estuary (Figure 3C). Fragments were the second most abundant shape, representing 27% and 41% of the MPs collected in the Douro and Lima estuaries, respectively (Figure 3C). On the other hand, films were less common, with only 11% in the Douro estuary and 8% in the Lima estuary (Figure 3C).

Regarding MP colours, blue was the most common in both estuaries, representing more than 50% of all MPs (65% in the Douro estuary and 61% in the Lima estuary) (Figure 3D). Ten more colours were detected in the MPs of the water samples (Figure 3D), namely red (Douro = 10%; Lima = 4.4%), pink (Douro = 1.4%; Lima = 3.7%), black (Douro = 1.8%;

Lima = 2.2%), orange (Douro = 1.6%; Lima = 5.8%), grey (Douro = 3.0%; Lima = 6.0%), and other colours with a vestigial representation in the samples (Figure 3D).

Regarding the MP size, smaller MPs (<1 mm) were the most abundant in both estuaries, representing 51% of all the MPs found in the Douro estuary and 61% in the Lima estuary (Figure 3E). MPs measuring between 1 mm and 3 mm comprised the second most abundant size class, accounting for 41% of all the MPs found in the Douro estuary and 36% in the Lima estuary. Finally, MPs larger than 3 mm were less common (Douro = 7.7%; Lima = 2.9%) (Figure 3E).

Regarding polymer identification in the water samples, an analysis was attempted on several MPs of varied sizes, colours, and particles. We were able to analyse and validate as MPs 10.4% of all MPs in our samples, which amounted to 127 MPs. These results are according to the recommendation of the European Union's Marine Strategy Framework Directive (MSFD) that a proportion of 10% of all samples should be routinely checked to confirm the accuracy of visual examination [46]. From the particles analysed, a total of seven different polymers were identified, including polyethylene (PE) and polypropylene (PP), which were the two most common. The other polymers identified were polyvinyl chloride (PVC), polyester (PET), polystyrene (PS), and poly(methyl methacrylate) (PMMA). Examples of the polymer analysis of water samples are shown in the Supplementary Materials (Figure S1).

3.3. MPs and Zooplankton

3.3.1. MP:Zooplankton Abundance Ratio

Overall, the estimated water MP:zooplankton ratio was 1:2.11 for the Douro estuary and 1:2.10 for the Lima estuary. In the two estuaries, there was a tendency for the ratios to increase over time, reaching higher values in November (Figure 4A). In fact, in November, the ratio reached 1:1.28 in the Douro estuary and 1:1.14 in the Lima estuary, indicating an increase in the MP concentration when compared to the zooplankton abundance.

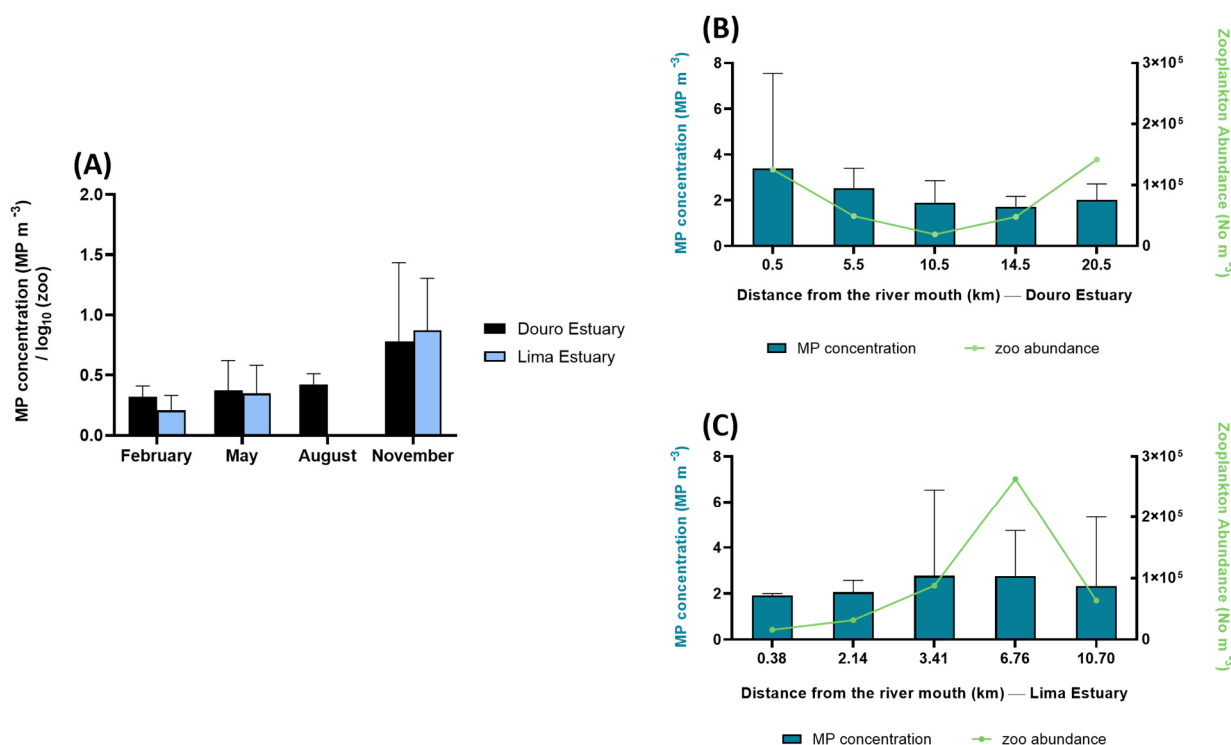


Figure 4. (A) Ratio between MP concentration in water samples and zooplankton abundance in Douro and Lima estuaries. Spatial variation in mean concentration of MPs in water samples and mean abundance of zooplankton in Douro (B) and Lima (C) estuaries.

Each estuary exhibited a specific temporal and spatial pattern of MP concentration and zooplankton abundance (Figure 4B,C).

In the Douro estuary, although no significant temporal or spatial patterns were observed, neither for zooplankton nor MPs, the zooplankton abundance tended to be lower in the middle estuary, while the MPs decreased with an increase in the distance from the river mouth. We also verified a trend for higher MPs in November, although it did not translate into significant variances (Figure 4B).

In the Lima estuary, the zooplankton abundance was significantly higher in the upper estuary, while the MP concentration did not vary significantly along the estuary. The temporal pattern revealed that the MP concentration was significantly higher in November, while the zooplankton abundance remained statically similar across all the sampling months (Figure 4C).

3.3.2. MPs in Zooplankton Organisms

No MP contamination was observed when dealing with zooplankton organisms.

From a total of 958 zooplankton organisms analysed (779 Copepoda and 179 Chaetognatha), 514 MPs were retrieved. In Copepoda, the MP contamination did not vary significantly between the two estuaries (one-way ANOVA: $F = 0.50$; $p \geq 0.05$), being on average 1.1 ± 1.2 MP ind⁻¹ and 0.91 ± 1.12 MP ind⁻¹ in the Douro and Lima estuaries, respectively. Similarly, the MP contamination in Chaetognatha did not vary significantly between the two estuaries (one-way ANOVA: $F = 2.78$; $p \geq 0.05$), but this last group was more contaminated with MPs, with average contamination levels of 1.98 ± 2.79 MP ind⁻¹ in the Douro estuary and 2.9 ± 3.1 MP ind⁻¹ in the Lima estuary.

While, in the Lima estuary, there were no significant spatial (Kruskal–Wallis: $H_4 = 43$; $p \geq 0.05$) (Figure 5D) or temporal (Kruskal–Wallis: $H_2 = 43$; $p \geq 0.05$) (Figure 5C) variations in the MP contamination in Copepoda, in the Douro estuary, significant differences were observed. The MP contamination of Copepoda in the Douro estuary varied significantly among the sampling stations (Kruskal–Wallis: $H_4 = 42$, $p < 0.05$), with sampling stations D1 and D4 presenting lower MP concentrations (Figure 5B). The MP contamination also varied significantly among months (one-way ANOVA: $F = 2.99$; $p < 0.05$), with the concentrations reaching their highest values in August (Figure 5A).

Regarding Chaetognatha, there were no significant spatial differences in the MP concentration, neither in the Douro estuary (one-way ANOVA: $F = 1.32$; $p \geq 0.05$) (Figure 5B) nor in the Lima estuary (Kruskal–Wallis: $H_4 = 18$, $p \geq 0.05$) (Figure 5D). In contrast, chaetognath contamination varied significantly among the sampling months in the Lima estuary (one-way ANOVA: $F = 5.68$; $p < 0.05$), with the concentration reaching its highest value in May, at 7.7 ± 5.7 MP ind⁻¹ (Figure 5C). In the Douro estuary, there were no significant differences among the sampling months (one-way ANOVA: $F = 0.83$; $p \geq 0.05$) (Figure 5D).

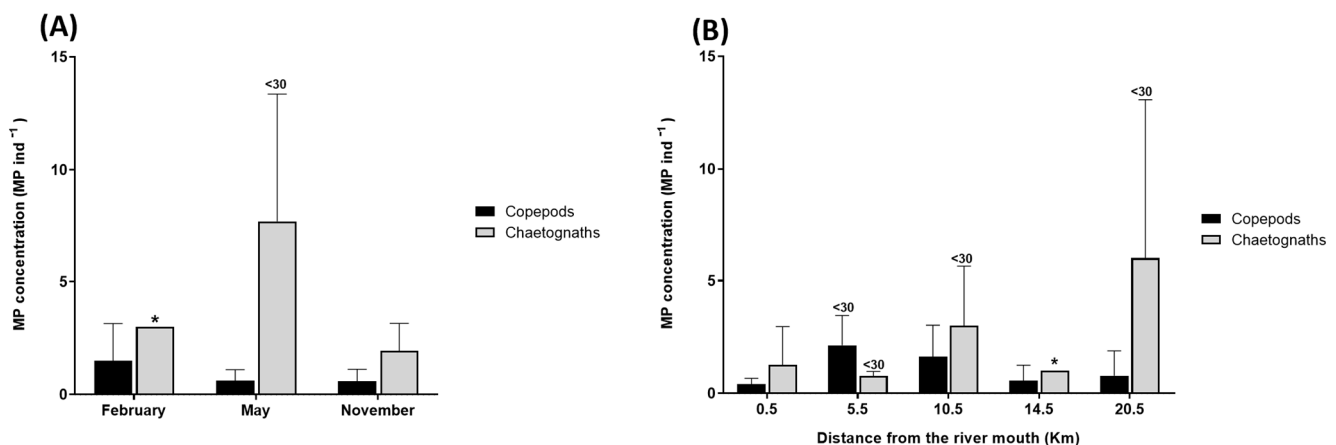
Regarding the MPs retrieved from Copepoda, fibres were the most common shape, both in the Douro estuary (55%) (Figure 6A) and in the Lima estuary (60%) (Figure 6D). Similarly, fibres were also the most frequent MP shape retrieved from chaetognaths of the Douro (52%) (Figure 6A) and Lima (58%) estuaries (Figure 6D). Fragments were the second most common MP shape retrieved from zooplanktonic organisms in both estuaries: 44% of the MPs retrieved in copepods and 47% of the MPs in Chaetognatha in the Douro estuary were fragments (Figure 6A), while in Lima, fragments amounted to 37% of all the MPs found in Copepoda and 40% in Chaetognatha (Figure 6D). Films were rarely observed, and the highest numbers were observed in Copepoda (1.79%) and Chaetognatha (1.71%), both from the Lima estuary (Figure 6D). In the Douro estuary, only chaetognaths were contaminated with films (0.65% of the MPs) (Figure 6A).

Blue was the most common colour of MPs found in all the zooplanktonic organisms. In the Douro estuary, 53% of the MPs found in Copepoda and 44% of the MPs in Chaetognatha were blue (Figure 6B). Similarly, in the Lima estuary, the majority of the MPs retrieved from zooplanktonic organisms were blue, namely 49% of the MPs in copepods and 43%

in Chaetognatha (Figure 6E). The remaining MPs were distributed between seven other colours (orange, green, red, black, white, grey, and transparent MPs).

In terms of size, the majority of the MPs found were from the smallest size class (smaller than 0.5 mm). Due to the high percentage of small MPs found in zooplankton when comparing to the results in the water samples, an extra size class (<0.5 mm) was considered in order to accurately represent the MPs in zooplankton. In the Douro estuary, this size class represented 80% of all the MPs found in Copepoda and 73% of the MPs in Chaetognatha (Figure 6C), while in the Lima estuary, 61% of the MPs found in copepods and 79% in Chaetognatha were in the smallest size class (Figure 6F). As the size of MPs increased, their overall percentage in the samples decreased, with larger MPs (>3 mm) accounting for only 3.5% of the MPs retrieved from Copepoda and 3.17% of Chaetognatha from the Douro estuary (Figure 6C), and 0.77% of the MPs found in copepods and 1.5.% of the MPs in Chaetognatha from the Lima estuary (Figure 6F).

Douro Estuary



Lima Estuary

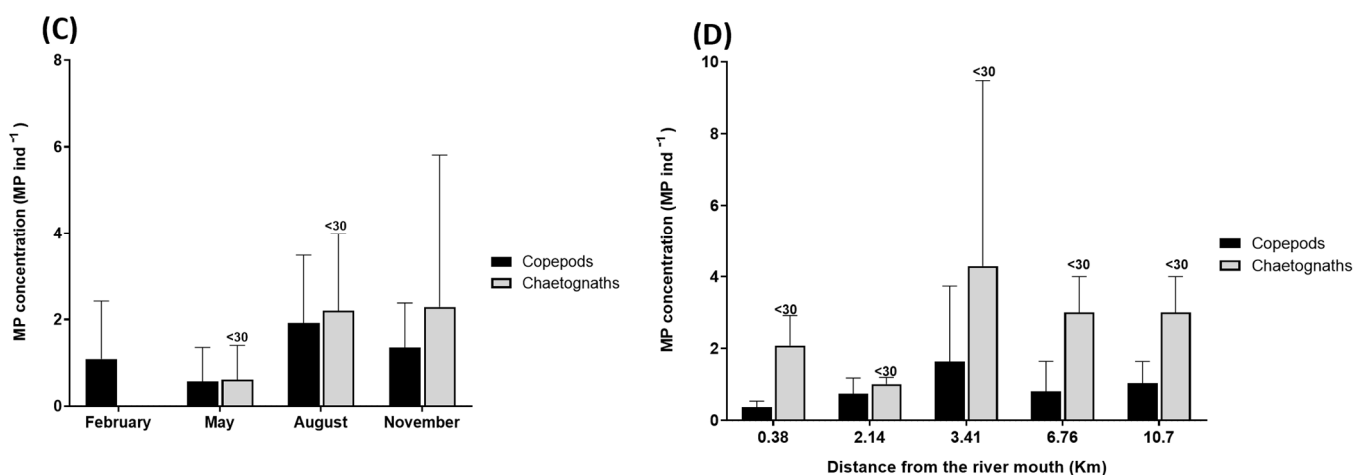


Figure 5. The concentrations of MPs in zooplanktonic organisms (copepods and chaetognaths) from the Douro estuary for different sampling months (A) and sampling stations (B), and from the Lima estuary for different months (C) and different sampling stations (D) (results are presented as means $\pm$ standard deviation; $n = 30$ except when number of organisms in the samples was lower; * indicates that only one individual (Chaetognatha) was analysed).

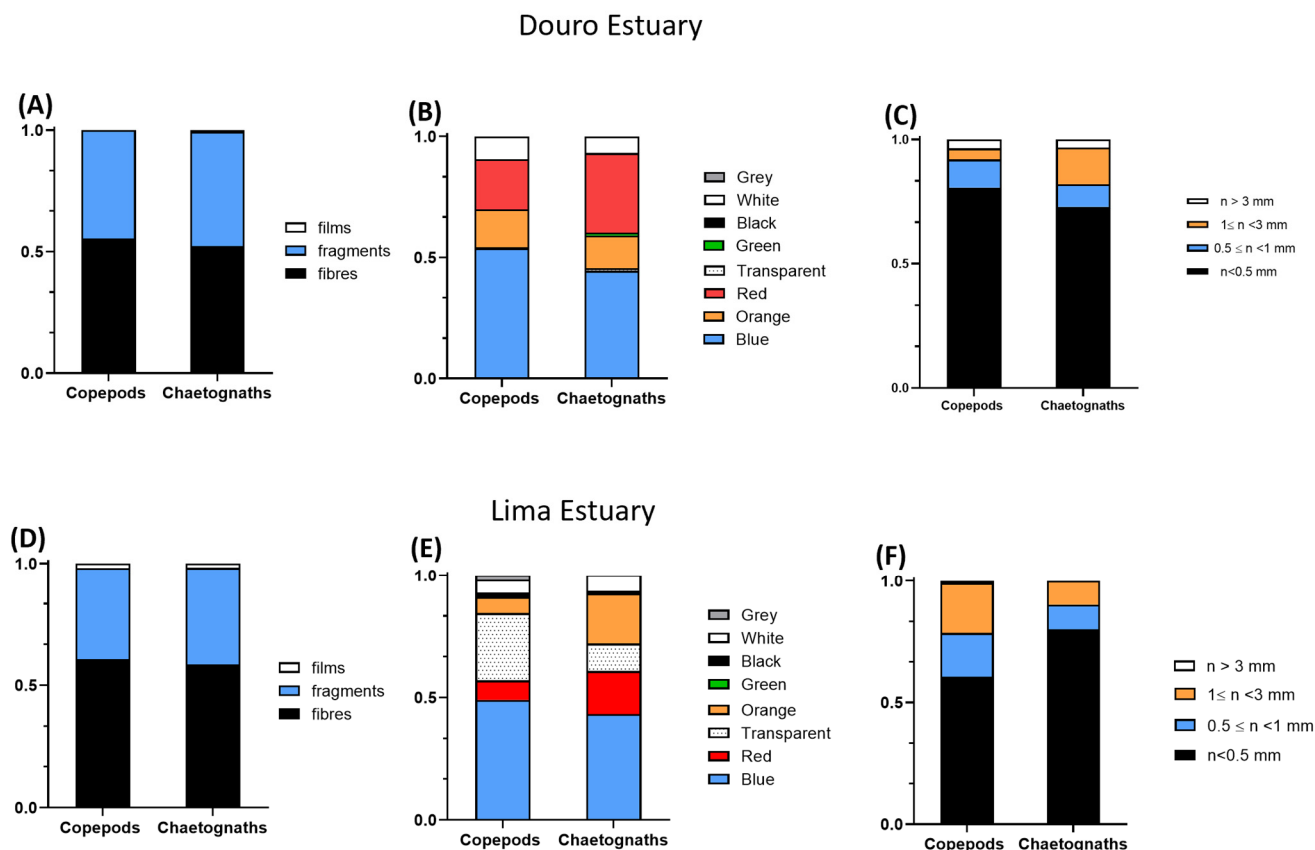


Figure 6. Characterization of MPs by shape (A,D), colour (B,E), and size (C,F) in copepods and chaetognaths, in the Douro estuary (A–C) and the Lima estuary (D–F).

Regarding polymer characterisation in the zooplanktonic samples, due to the small dimensions of these MPs (the majority being thin fibres smaller than 0.5 mm) and the characteristics of the FTIR equipment available, only one MP, a blue fibre, provided a match that complied with our acceptance criteria (matched with the spectra library by $\geq 75\%$); it was identified as polypropylene (PP) with a spectral match of 96.5% when compared to the library spectra (Figure S2).

4. Discussion

MP contamination is an emergent concern in aquatic environments, and although research on this topic has been increasing recently, there is still missing information from field studies on MP contamination levels and how MPs impact environments and wildlife, in particular organisms on lower trophic levels of the food web, such as plankton. In Portugal, namely in the Douro and Lima estuaries, there are still few studies regarding MP contamination, namely those conducted by Rodrigues et al. [17] and Prata et al. [48] in the Douro estuary and Almeida et al. [49] in the Lima estuary.

The present study is the first to investigate a possible relationship between MPs and zooplankton in two distinct estuaries, the Douro estuary and the Lima estuary, and provide important field insights into the zooplankton ingestion of MPs and the possible trophic transfer of MPs in the food web.

4.1. MP Contamination in Zooplankton in the Douro and Lima Estuaries

This study is, to the best of our knowledge, the first to show the MP contamination of both Copepoda and Chaetognatha in the Douro and Lima estuaries and one of the very few studies on MPs in these organisms at the base of the food web. The MP concentration in Copepoda and Chaetognatha in both estuaries had the same order of magnitude.

Overall, field studies regarding MPs in copepods and chaetognaths are scarce, and the concentrations found in our study were slightly higher than the values found in other studies with field samples. For example, Kosore et al. [50] found Chaetognatha to have ingested 0.46 particles ind⁻¹ and copepods 0.33 particles ind⁻¹, while Sipps et al. [9] reported concentrations between 0.30 and 0.82 MPs individual⁻¹ for three different species of copepods. It is important to note that each of these studies used different methodologies, and therefore, comparisons should be made cautiously. Furthermore, Sipps et al. [9] noted in their study that the nitric acid used for the isolation of ingested MPs could cause the depolymerisation and fragmentation of certain polymers, undervaluing the total value of the MPs ingested. Due to the optimization and validation of our protocol for MPs in zooplankton organisms [43], we ensured that we did not underestimate the MP values, because our protocol enabled the proper degradation of the zooplankton organisms while maintaining the polymerisation and physical integrity of all the MPs in the samples.

The results showed that, in both estuaries, Chaetognatha exhibited higher MP contamination levels than Copepoda. The differences between the MP concentration in Copepoda (Douro = 1.1 ± 1.2 MP ind⁻¹, Lima = 0.91 ± 1.12 MP ind⁻¹) and Chaetognatha (Douro = 2.0 ± 2.8 MP ind⁻¹, Lima = 2.9 ± 3.1 MP ind⁻¹) may be a consequence of the higher body size of Chaetognatha, and therefore, a higher concentration of MPs could be expected. However, it is important to take into consideration the trophic levels of these two types of zooplankton and their distinct feeding habits: Copepoda are mostly primary consumers, feeding on phytoplankton and protists, while Chaetognatha are considered secondary consumers, feeding on zooplankton such as Copepoda [44,45,50]. Therefore, the present results indicate a potential MP transfer between these two trophic levels, as Chaetognatha may feed on contaminated Copepoda. Similar hypotheses were noted by Goswami et al. [51] and Sun et al. [22], who reported that zooplankton in a higher trophic level are more susceptible to accidental MP ingestion or accumulation due to contaminated prey. Hence, the present results support MP transfer along the trophic chain, highlighting the fact that it can start at the lower levels of the trophic chain.

Regarding the characterization of the MPs found in zooplankton organisms, the majority of the MPs found in Copepoda and Chaetognatha were fibres. These results are in accordance with the results of the water samples. In fact, in the Douro and Lima estuarine water, fibres were the most common MP shape observed in these aquatic environments, and consequently, they were more easily accessible for ingestion by zooplankton. Similar results were reported by Sun et al. [22], who found fibres to be the most common MP shape in seawater and in different zooplankton taxa—fibres represented 59.0% of the MPs found in Copepoda and 56.4% in Chaetognatha. Likewise, Klasios and Tseng [25] also found fibres to be the most common MPs in subsurface lake waters and in Copepoda. Furthermore, the authors pointed out that, due to the small width of fibres and their ability to be twisted and folded to smaller sizes, their bioavailability increases, as they are more easily ingested by small organisms such as zooplankton.

It is worth noting that, although there has been a recent upsurge of studies on the ingestion and impacts of MPs in smaller marine organisms such as zooplankton, the majority of these studies have been performed under laboratory conditions, often using virgin MPs in higher concentrations than the ones commonly registered in field studies. Furthermore, as highlighted by Rodrigues et al. [24], laboratory studies tend to use spherical MPs (beads) in their experiments, which represents a MP shape that is not commonly reported by field studies. Despite the importance of these studies in understanding the biological impacts of MP ingestion on zooplankton health, such as internal injuries [23,26,27], impaired feeding behaviour, and reduced fecundity [28], it is critical to recognize that the damages reported in these laboratory settings may not accurately reflect the impacts of MPs on zooplankton in the field, and that field studies regarding this topic are often limited and fewer than their laboratory counterparts. Hence, our results support the necessity of the use of realistic MP concentrations and shapes, such as fibres, as well as MPs in different fragmentation and degradation states, in laboratory experiments to properly assess the ingestion of MPs

by zooplankton. On the other hand, we reinforce the need for further field studies to increase the scientific knowledge of how organisms are contaminated by MPs under realistic conditions. Therefore, our study aimed to assess the concentration of MPs in zooplankton organisms in their natural environment, either by the direct ingestion of MPs or by the ingestion of prey contaminated by MPs.

In terms of MP colours, blue was the most common colour found in Copepoda and Chaetognatha. This might be a consequence of the fact that the MPs from the surrounding water were also mostly blue, and due to the resemblance of blue MPs to typical food colours. Several studies have also reported blue as the most common colour, such as Goswami et al. [51], the authors of which determined that blue MPs represent 50% of all the MPs found in zooplankton. Trindade et al. (2023) points to blue pigment stability as a possible reason for the high numbers and persistence of blue plastics in the environment [10]. Many blue pigments, such as indigo blue (widely used in the textile industry, especially in denim manufacturing), have a high colour stability and a high resistance to heat and light [10,52]. In particular, blue fibres from denim manufacturing have been identified as a major indicator of anthropogenic pollution [53]. On the other hand, blue MPs may be prevalent due to the degradation of blue fishing lines [51].

In terms of size, there was also a tendency for the majority of the MPs found in zooplanktonic organisms to be from the smallest size class considered, i.e., below 0.5 mm. Several other studies have also observed the same tendency for smaller fragments to be more bioavailable for zooplankton [25].

The present study indicates that the zooplankton of the two estuaries might be under similar pressures posed by MPs, since the level of MP contamination of zooplanktonic organisms was similar between the two estuaries. Moreover, a similar average ratio between MPs and zooplankton was observed. The two estuaries also showed a similar temporal tendency for these ratios to increase in autumn (November sampling), which was associated with an increasing MP contamination level in the water. Due to the importance of zooplankton to the food web and complex ecosystems such as estuaries, it is important to study the threats and interferences to these organisms. The ingestion of MPs by Copepoda may be one of the entry points of MPs into the food web, and one of the first transfers of MPs through trophic levels, from primary consumers (Copepoda) to secondary consumers (Chaetognatha) that feed on contaminated Copepoda.

4.2. MP's Presence in Estuarine Water

The present study confirmed the contamination of the Douro and Lima estuaries with MPs, with similar contamination levels (2.3 ± 1.9 MP m⁻³ in the Douro estuary and 2.4 ± 2.0 MP m⁻³ in the Lima estuary). A previous study by Rodrigues et al. [17] also characterized the MPs in water samples from the Douro estuary, and found levels of 0.17 MPs m⁻³, while Prata et al. [48] found a median MP concentration of 0.23 MP m⁻³ among three different sampling areas in the Douro estuary: a countryside area; a wastewater treatment effluent release zone; and an area in proximity to a boat dock and maintenance station. In the Lima estuary, Almeida et al. [49] found MP concentrations of estuarine water ranging from 0.010 MP m⁻³ to 0.20 MP m⁻³. Among other estuaries and enclosed water forms, our results are within the same range found at a Turkish river mouth in the Black Sea (3.3 ± 2.0 particles m⁻³) by Aytan et al. [54], and at the Adour estuary, in France [8]. However, other studies have shown MP concentrations in higher orders of magnitude, such as Taha et al. [18], the authors of which retrieved 1687 particles m⁻³ from the Terengganu estuary, in Malaysia; or Trindade et al. [10] in a heavily populated bay in Brazil (5180 items m⁻³). Likewise, lower MP concentrations have also been reported, namely by Lima et al. [16] (0.2604 items m⁻³) or Sun et al. [21], who reported MP concentrations of 0.13 ± 0.20 items m⁻³ in the Yellow Sea. It is important to highlight that differences in the sampling methods, such as different net sizes, water pumps, and depths at which the samples are retrieved, as well as the wide range of different protocols used for

processing MPs, can influence the results, and should be taken into consideration when drawing comparisons.

When comparing both estuaries in our study, we initially hypothesized that the Douro estuary would present higher MP contamination than the Lima estuary, since the Douro estuary is more impacted [38], namely by a heavier urban pressure due to the presence of two major cities in the vicinity of the estuary (Porto and Vila Nova de Gaia). Several studies [21,55,56] have related the proximity to large urban centres and hotspots of human activities as major sources of MP contamination. However, the results showed that the MP concentration in the water samples of both estuaries was similar. Gray et al. [14], when comparing two South Carolina estuaries, also faced the same results, in which the estuary with the lowest surrounding population was revealed to be the most contaminated, with a higher MP concentration. This outcome could be related to the features of the total area and the drainage area of the estuaries, such as anthropogenic pressure and industrial activities, showcasing that these factors could be more prone to influencing the MP concentration than the immediate surrounding population and subsequent human activity [14]. In fact, our results showed that, in November, the MP concentration increased in both estuaries, specifically in the Lima estuary, where the MP contamination was higher on average than in the Douro estuary. Such an increase in the MP contamination could have been associated with higher precipitation values, which are typical of this time of the year (heavy rain in the days prior to our sampling campaigns were observed), that might have transported MPs from upstream locations and river banks to the estuaries [17]. Hence, the MP contamination dynamics in transitional ecosystems such as estuaries are complex and influenced by the temporal and spatial hydrodynamics of each estuary.

Regarding the characterization of the MPs found in the water samples, we verified similar results between estuaries, with fibres being the most common shape of MPs, followed by fragments and films. Rodrigues et al. [17] in the Douro estuary and Almeida et al. [49] in the Lima estuary also reported fibres as being the most prevalent shape of MPs in water samples. Several studies have linked the presence of fibres in aquatic environments to domestic sewage and the proximity to WWTPs [14,57], whose processes are often proven to be inefficient in the removal of microfibres from water [58,59]. Viitala et al. (2022) [60] and Surana et al. (2024) [61] have also reported an increase in the amount of textile fibres in sediments and effluents from WWTPs. Fibres are also associated with the remnants of fishing gear and other maritime activities (fishing lines, nets, and ropes) [51]. Although different, the two estuaries are exposed to important fibre sources, namely WWTPs and touristic maritime activities in the Douro estuary [17] and maritime activities such as maritime transport, fishing, and aquaculture facilities in the Lima estuary. A variety of differently coloured MPs was also observed, with blue representing the most common colour, both in the Douro estuary and the Lima estuary. Overall, blue is regarded as one of the most common colours of MPs detected in aquatic environments [10]. The noticeable presence of polyethylene in both estuaries might be associated with the proximity to urban centres and areas with a heavy tourist pressure, since polyethylene is a common plastic polymer used to produce containers, wrappings, and plastic bags. The second most common polymer found in our study was polypropylene, which, despite all other potential domestic sources, can be associated with maritime activities, namely fishing gear, netting, rope, and bottle caps [62,63].

5. Conclusions

The present study showed the presence of MPs in the water and zooplankton from two Portuguese estuaries, the Douro estuary and the Lima estuary, with an average ratio of 1 MP:2.11 zooplankton organisms for the Douro estuary and 1 MP:2.10 zooplankton organisms for the Lima estuary. Copepoda and Chaetognatha from the two estuaries were contaminated with MPs, with similar values between the estuaries. The results indicate that zooplankton organisms are mainly contaminated by similar MPs, comprising blue fibres of a small size, which coincide with the MPs most commonly found in their

surrounding environment (water). Notably, Chaetognatha were more contaminated with MPs than Copepoda, possibly indicating the trophic transfer of MPs through the food web by the ingestion of contaminated Copepoda by Chaetognatha. Overall, our study provides further understanding of MP contamination in estuarine environments, and gives important insights about the ingestion of MPs by Copepoda and Chaetognatha. We can account for a possible trophic transfer of MPs in the food web. Our study highlights the need to further investigate the ingestion of MPs by zooplankton and its impact on these organisms, as well as the impact on the ecosystems they inhabit.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w16070992/s1>, Figure S1: FTIR analysis of MPs from water samples; Figure S2: FTIR analysis of MPs from zooplanktonic samples.

Author Contributions: Conceptualization, F.E., C.M.R.A. and S.R.; methodology, F.E., R.P., S.M.R. and D.M.S.; validation, C.M.R.A. and S.R.; formal analysis, F.E. and R.P.; investigation, F.E., R.P., S.M.R., D.M.S., C.M.R.A. and S.R.; resources, C.M.R.A. and S.R.; data curation, F.E., C.M.R.A. and S.R.; writing—original draft preparation, F.E.; writing—review and editing, F.E., C.M.R.A. and S.R.; supervision, C.M.R.A. and S.R.; project administration, C.M.R.A. and S.R.; funding acquisition, C.M.R.A. and S.R. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the project Ocean3R (NORTE-01-0145-FEDER-000064), supported by the North Portugal Regional Operational Programme (NORTE2020) under the PORTUGAL 2020 Partnership Agreement and through the European Regional Development Fund (ERDF). Financial support was also received from the FCT Foundation for Science and Technology, within the scope of UIDB/04423/2020 and UIDP/04423/2020.

Data Availability Statement: The data will be provided upon request.

Acknowledgments: The authors acknowledge Fundação para a Ciência e Tecnologia (FCT) for the PhD scholarships to S.M.R. (SFRH/BD/145736/2019), D.M.S. (2020.06088.BD), and R.P. (2021.04850.BD), and a research contract to S.R. (DL57/2016/CP1344/CT0020).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. UNEP. *Marine Plastic Debris and Microplastics—Global Lessons and Research To Inspire Action and Guide Policy Change*; United Nations Environment Programme: Nairobi, Kenya, 2016.
2. Eriksen, M.; Lebreton, L.C.M.; Carson, H.S.; Thiel, M.; Moore, C.J.; Borerro, J.C.; Galgani, F.; Ryan, P.G.; Reisser, J. Plastic Pollution in the World's Oceans: More than 5 Trillion Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. *PLoS ONE* **2014**, *9*, e111913. [\[CrossRef\]](#)
3. Arthur, C.J.; Baker, J.; Bamford, H. (Eds.) Small Plastic Debris and Plankton: Perspectives from NOAA Plankton Sampling Programs in Northeast Pacific Ecosystems. In Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris, Washington, DC, USA, 9–11 September 2008; NOAA Technical Memorandum NOS-OR&R-30. 2009.
4. Herrera, A.; Asensio, M.; Martínez, I.; Santana, A.; Packard, T.; Gómez, M. Microplastic and tar pollution on three Canary Islands beaches: An annual study. *Mar. Pollut. Bull.* **2018**, *129*, 494–502. [\[CrossRef\]](#)
5. Karlsson, T.M.; Vethaak, A.D.; Almroth, B.C.; Ariese, F.; van Velzen, M.; Hassellöv, M.; Leslie, H.A. Screening for microplastics in sediment, water, marine invertebrates and fish: Method development and microplastic accumulation. *Mar. Pollut. Bull.* **2017**, *122*, 403–408. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Horton, A.A.; Walton, A.; Spurgeon, D.J.; Lahive, E.; Svendsen, C. Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities. *Sci. Total. Environ.* **2017**, *586*, 127–141. [\[CrossRef\]](#)
7. Xiao, S.; Cui, Y.; Brahney, J.; Mahowald, N.M.; Li, Q. Long-distance atmospheric transport of microplastic fibres influenced by their shapes. *Nat. Geosci.* **2023**, *16*, 863–870. [\[CrossRef\]](#)
8. Defontaine, S.; Sous, D.; Tesan, J.; Monperrus, M.; Lenoble, V.; Lanceleur, L. Microplastics in a salt-wedge estuary: Vertical structure and tidal dynamics. *Mar. Pollut. Bull.* **2020**, *160*, 111688. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Sipps, K.; Arbuckle-Keil, G.; Chant, R.; Fahrenfeld, N.; Garzio, L.; Walsh, K.; Saba, G. Pervasive occurrence of microplastics in Hudson-Raritan estuary zooplankton. *Sci. Total Environ.* **2022**, *817*, 152812. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Trindade, L.d.S.; Gloaguen, T.V.; Benevides, T.d.S.F.; Valentim, A.C.S.; Bomfim, M.R.; Santos, J.A.G. Microplastics in surface waters of tropical estuaries around a densely populated Brazilian bay. *Environ. Pollut.* **2023**, *323*, 121224. [\[CrossRef\]](#) [\[PubMed\]](#)

11. Murphy, F.; Ewins, C.; Carbonnier, F.; Quinn, B. Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment. *Environ. Sci. Technol.* **2016**, *50*, 5800–5808. [\[CrossRef\]](#)
12. Barnes, D.K.A.; Galgani, F.; Thompson, R.C.; Barlaz, M. Accumulation and fragmentation of plastic debris in global environments. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2009**, *364*, 1985–1998. [\[CrossRef\]](#)
13. Do, H.-L.; Armstrong, C.W. Ghost fishing gear and their effect on ecosystem services—Identification and knowledge gaps. *Mar. Policy* **2023**, *150*, 105528. [\[CrossRef\]](#)
14. Gray, A.D.; Wertz, H.; Leads, R.R.; Weinstein, J.E. Microplastic in two South Carolina Estuaries: Occurrence, distribution, and composition. *Mar. Pollut. Bull.* **2018**, *128*, 223–233. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Browne, M.A.; Galloway, T.S.; Thompson, R.C. Spatial Patterns of Plastic Debris along Estuarine Shorelines. *Environ. Sci. Technol.* **2010**, *44*, 3404–3409. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Lima, A.; Costa, M.; Barletta, M. Distribution patterns of microplastics within the plankton of a tropical estuary. *Environ. Res.* **2014**, *132*, 146–155. [\[CrossRef\]](#) [\[PubMed\]](#)
17. Rodrigues, S.; Almeida, C.M.R.; Silva, D.; Cunha, J.; Antunes, C.; Freitas, V.; Ramos, S. Microplastic contamination in an urban estuary: Abundance and distribution of microplastics and fish larvae in the Douro estuary. *Sci. Total Environ.* **2018**, *659*, 1071–1081. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Taha, Z.D.; Amin, R.M.; Anuar, S.T.; Nasser, A.A.A.; Sohaimi, E.S. Microplastics in seawater and zooplankton: A case study from Terengganu estuary and offshore waters, Malaysia. *Sci. Total Environ.* **2021**, *786*, 147466. [\[CrossRef\]](#)
19. NOAA. What Are Plankton? Available online: <https://oceanservice.noaa.gov/facts/plankton.html> (accessed on 19 June 2022).
20. Havens, K.E. Zooplankton Structure and Potential Food Web Interactions in the Plankton of a Subtropical Chain-of-Lakes. *Sci. World J.* **2002**, *2*, 926–942. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Sun, X.; Liang, J.; Zhu, M.; Zhao, Y.; Zhang, B. Microplastics in seawater and zooplankton from the Yellow Sea. *Environ. Pollut.* **2018**, *242*, 585–595. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Sun, X.; Liu, T.; Zhu, M.; Liang, J.; Zhao, Y.; Zhang, B. Retention and characteristics of microplastics in natural zooplankton taxa from the East China Sea. *Sci. Total Environ.* **2018**, *640–641*, 232–242. [\[CrossRef\]](#)
23. Wright, S.L.; Thompson, R.C.; Galloway, T.S. The physical impacts of microplastics on marine organisms: A review. *Environ. Pollut.* **2013**, *178*, 483–492. [\[CrossRef\]](#)
24. Rodrigues, S.M.; Elliott, M.; Almeida, C.M.R.; Ramos, S. Microplastics and plankton: Knowledge from laboratory and field studies to distinguish contamination from pollution. *J. Hazard. Mater.* **2021**, *417*, 126057. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Klasios, N.; Tseng, M. Microplastics in subsurface water and zooplankton from eight lakes in British Columbia. *Can. J. Fish. Aquat. Sci.* **2023**, *80*, 1248–1267. [\[CrossRef\]](#)
26. Zavala-Alarcón, F.L.; Huchin-Mian, J.P.; González-Muñoz, M.D.P.; Kozak, E.R. In situ microplastic ingestion by neritic zooplankton of the central Mexican Pacific. *Environ. Pollut.* **2023**, *319*, 120994. [\[CrossRef\]](#) [\[PubMed\]](#)
27. He, M.; Yan, M.; Chen, X.; Wang, X.; Gong, H.; Wang, W.; Wang, J. Bioavailability and toxicity of microplastics to zooplankton. *Gondwana Res.* **2021**, *108*, 120–126. [\[CrossRef\]](#)
28. Cole, M.P.; Lindeque, P.; Fileman, E.; Halsband, C.; Galloway, T.S. The Impact of Polystyrene Microplastics on Feeding, Function and Fecundity in the Marine Copepod *Calanus helgolandicus*. *Environ. Sci. Technol.* **2015**, *49*, 1130–1137. [\[CrossRef\]](#)
29. Zhang, C.; Jeong, C.-B.; Lee, J.-S.; Wang, D.-Z.; Wang, M. Transgenerational Proteome Plasticity in Resilience of a Marine Copepod in Response to Environmentally Relevant Concentrations of Microplastics. *Environ. Sci. Technol.* **2019**, *53*, 8426–8436. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Cole, M.; Lindeque, P.; Fileman, E.; Halsband, C.; Goodhead, R.; Moger, J.; Galloway, T.S. Microplastic Ingestion by Zooplankton. *Environ. Sci. Technol.* **2013**, *47*, 6646–6655. [\[CrossRef\]](#)
31. Vroom, R.J.; Koelmans, A.A.; Besseling, E.; Halsband, C. Aging of microplastics promotes their ingestion by marine zooplankton. *Environ. Pollut.* **2017**, *231*, 987–996. [\[CrossRef\]](#)
32. Setälä, O.; Fleming-Lehtinen, V.; Lehtiniemi, M. Ingestion and transfer of microplastics in the planktonic food web. *Environ. Pollut.* **2014**, *185*, 77–83. [\[CrossRef\]](#)
33. Azevedo, I.C.; Duarte, P.M.; Bordalo, A.A. Understanding spatial and temporal dynamics of key environmental characteristics in a mesotidal Atlantic estuary (Douro, NW Portugal). *Estuarine Coast. Shelf Sci.* **2008**, *76*, 620–633. [\[CrossRef\]](#)
34. Vieira, M.E.; A Bordalo, A. The Douro estuary (Portugal): A mesotidal salt wedge. *Oceanol. Acta* **2000**, *23*, 585–594. [\[CrossRef\]](#)
35. Azevedo, I.C.; Duarte, P.M.; Bordalo, A.A. Pelagic metabolism of the Douro estuary (Portugal)—Factors controlling primary production. *Estuarine Coast. Shelf Sci.* **2006**, *69*, 133–146. [\[CrossRef\]](#)
36. Ramos, S.; Ré, P.; Bordalo, A.A. Recruitment of flatfish species to an estuarine nursery habitat (Lima estuary, NW Iberian Peninsula). *J. Sea Res.* **2010**, *64*, 473–486. [\[CrossRef\]](#)
37. Costa-Dias, S.; Sousa, R.; Antunes, C. Ecological quality assessment of the lower Lima Estuary. *Mar. Pollut. Bull.* **2010**, *61*, 234–239. [\[CrossRef\]](#) [\[PubMed\]](#)
38. Ramos, S.; Cabral, H.; Elliott, M. Do fish larvae have advantages over adults and other components for assessing estuarine ecological quality? *Ecol. Indic.* **2015**, *55*, 74–85. [\[CrossRef\]](#)
39. Berraho, A.; Somoue, L.; Hernández-León, S.; Valdés, L. Zooplankton in the Canary Current Large Marine Ecosystem. In *Oceanographic and Biological Features in the Canary Current Large Marine Ecosystem*; Valdés, L., Déniz-González, I., Eds.; IOC-UNESCO: Paris, France, 2015; pp. 183–195.

40. Cornils, A.K.; Thomisch, K.; Hase, J.; Hildebrandt, N.; Auel, H.; Niehoff, B. Testing the usefulness of optical data for zooplankton long-term monitoring: Taxonomic composition, abundance, biomass, and size spectra from ZooScan image analysis. *Limnol. Oceanogr. Methods* **2022**, *20*, 428–450. [\[CrossRef\]](#)
41. Pereira, R.; Rodrigues, S.M.; Silva, D.M.; Ramos, S. Assessing Environmental Control on Temporal and Spatial Patterns of Larval Fish Assemblages in a Marine Protected Area. *Ecologies* **2023**, *4*, 288–309. [\[CrossRef\]](#)
42. Rodrigues, S.; Almeida, C.M.R.; Ramos, S. Adaptation of a laboratory protocol to quantify microplastics contamination in estuarine waters. *MethodsX* **2019**, *6*, 740–749. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Rodrigues, S.; Espincho, F.; Elliott, M.; Almeida, C.M.R.; Ramos, S. Methodology optimization to quantify microplastic presence in planktonic copepods, chaetognaths and fish larvae. *MethodsX* **2023**, *11*, 102466. [\[CrossRef\]](#)
44. Baier, C.; Purcell, J. Trophic interactions of chaetognaths, larval fish, and zooplankton in the South Atlantic Bight. *Mar. Ecol. Prog. Ser.* **1997**, *146*, 43–53. [\[CrossRef\]](#)
45. Terazaki, M. Life history, distribution, seasonal variability and feeding of the pelagic chaetognath *Sagitta elegans* in the Subarctic Pacific: A review. *Plankton Biol. Ecol.* **1998**, *45*, 1–17.
46. European Commission and Joint Research Centre. *Guidance on the Monitoring of Marine Litter in European Seas—An update to Improve the Harmonised Monitoring of Marine Litter under the Marine Strategy Framework Directive*; Publications Office of the European Union: Luxembourg, 2023.
47. Rodrigues, S.; Almeida, C.M.R.; Ramos, S. Microplastics contamination along the coastal waters of NW Portugal. *Case Stud. Chem. Environ. Eng.* **2020**, *2*, 100056. [\[CrossRef\]](#)
48. Prata, J.C.; Godoy, V.; da Costa, J.P.; Calero, M.; Martín-Lara, M.; Duarte, A.C.; Rocha-Santos, T. Microplastics and fibers from three areas under different anthropogenic pressures in Douro river. *Sci. Total Environ.* **2021**, *776*, 145999. [\[CrossRef\]](#)
49. Almeida, C.M.R.; Sáez-Zamacona, I.; Silva, D.M.; Rodrigues, S.M.; Pereira, R.; Ramos, S. The Role of Estuarine Wetlands (Saltmarshes) in Sediment Microplastics Retention. *Water* **2023**, *15*, 1382. [\[CrossRef\]](#)
50. Kosore, C.L.; Ojwang, L.; Maghanga, J.; Kamau, J.; Kimeli, A.; Omukoto, J.; Ngisiani, N.; Mwaluma, J.; Ong'ada, H.; Magori, C.; et al. Occurrence and ingestion of microplastics by zooplankton in Kenya's marine environment: First documented evidence. *Afr. J. Mar. Sci.* **2018**, *40*, 225–234. [\[CrossRef\]](#)
51. Goswami, P.; Selvakumar, N.; Verma, P.; Saha, M.; Suneel, V.; Vinithkumar, N.V.; Dharani, G.; Rathore, C.; Nayak, J. Microplastic intrusion into the zooplankton, the base of the marine food chain: Evidence from the Arabian Sea, Indian Ocean. *Sci. Total Environ.* **2023**, *864*, 160876. [\[CrossRef\]](#)
52. Imhof, H.K.; Laforsch, C.; Wiesheu, A.C.; Schmid, J.; Anger, P.M.; Niessner, R.; Ivleva, N.P. Pigments and plastic in limnetic ecosystems: A qualitative and quantitative study on microparticles of different size classes. *Water Res.* **2016**, *98*, 64–74. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Athey, S.N.; Adams, J.K.; Erdle, L.M.; Jantunen, L.M.; Helm, P.A.; Finkelstein, S.A.; Diamond, M.L. The Widespread Environmental Footprint of Indigo Denim Microfibers from Blue Jeans. *Environ. Sci. Technol. Lett.* **2020**, *7*, 840–847. [\[CrossRef\]](#)
54. Aytan, U.; Esensoy, F.B.; Senturk, Y. Microplastic ingestion and egestion by copepods in the Black Sea. *Sci. Total Environ.* **2022**, *806*, 150921. [\[CrossRef\]](#)
55. Desforges, J.-P.W.; Galbraith, M.; Ross, P.S. Ingestion of Microplastics by Zooplankton in the Northeast Pacific Ocean. *Arch. Environ. Contam. Toxicol.* **2015**, *69*, 320–330. [\[CrossRef\]](#)
56. Tibbetts, J.; Krause, S.; Lynch, I.; Smith, G.H.S. Abundance, Distribution, and Drivers of Microplastic Contamination in Urban River Environments. *Water* **2018**, *10*, 1597. [\[CrossRef\]](#)
57. Napper, I.E.; Thompson, R.C. Release of synthetic microplastic plastic fibres from domestic washing machines: Effects of fabric type and washing conditions. *Mar. Pollut. Bull.* **2016**, *112*, 39–45. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Montecinos, S.; Gil, M.; Tognana, S.; Salgueiro, W.; Amalvy, J. Distribution of microplastics present in a stream that receives discharge from wastewater treatment plants. *Environ. Pollut.* **2022**, *314*, 120299. [\[CrossRef\]](#)
59. Sun, J.; Dai, X.; Wang, Q.; van Loosdrecht, M.C.; Ni, B.-J. Microplastics in wastewater treatment plants: Detection, occurrence and removal. *Water Res.* **2019**, *152*, 21–37. [\[CrossRef\]](#) [\[PubMed\]](#)
60. Viitala, M.; Steinmetz, Z.; Sillanpää, M.; Mänttari, M.; Sillanpää, M. Historical and current occurrence of microplastics in water and sediment of a Finnish lake affected by WWTP effluents. *Environ. Pollut.* **2022**, *314*, 120298. [\[CrossRef\]](#) [\[PubMed\]](#)
61. Surana, D.; Vinay, P.; Ghosh, P.; Sharma, S.; Kumar, V.; Kumar, S. Microplastic Fibers in Different Environmental Matrices from Synthetic Textiles: Ecotoxicological Risk, Mitigation Strategies, and Policy Perspective. *J. Environ. Chem. Eng.* **2024**, *12*, 112333. [\[CrossRef\]](#)
62. Coyle, R.; Hardiman, G.; Driscoll, K.O. Microplastics in the marine environment: A review of their sources, distribution processes, uptake and exchange in ecosystems. *Case Stud. Chem. Environ. Eng.* **2020**, *2*, 100010. [\[CrossRef\]](#)
63. Gesamp, G. *Sources, Fate and Effects of Microplastics in the Marine Environment: Part Two of a Global Assessment*; IMO: London, UK, 2016; p. 220.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.